.

AD_____

Award Number: DAMD17-02-1-0076

TITLE: Enzymatic Activation of Peptide Prodrugs by Prostate-Specific Membrane Antigen (PSMA) as Targeted Therapy for Prostate Cancer

PRINCIPAL INVESTIGATOR: Samuel R. Denmeade, M.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
Baltimore, MD 21205-2196

REPORT DATE: January 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-01-2005	Final	1 JAN 2004 - 31 DEC 2004

4. TITLE AND SUBTITLE
Enzymatic Activation of Peptide Prodrugs by Prostate-Specific Membrane Antigen (PSMA) as Targeted Therapy for Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-02-1-0076

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Samuel R. Denmeade, M.D.

E-Mail: denmesa@jhmi.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Johns Hopkins University
Baltimore, MD 21231

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: The majority of our present chemotherapeutic agents only kill cells effectively when they are proliferating; this may explain why these agents have been of such limited success in patients. In contrast to these ineffective agents, we have chemically modified a plant toxin, Thapsigargin (TG), to produce primary amine-containing analogs that are potent, cell proliferation independent, inducers of apoptosis in prostate cancer cells. These TG-analogs, however, are not prostate cancer-specific cytotoxins. The hypothesis is that a potent TG analog can be converted to an inactive prodrug by coupling to a peptide carrier that is a substrate for Prostate Specific Membrane Antigen (PSMA). Since PSMA is expressed in high levels only by prostate cancer cells and not by normal cells, this should allow specific targeting of the TG-analog's killing ability to prostate cancer cells thus minimizing toxicity to normal tissue. Two enzymatic activities for PSMA have been described: an N-acetyl-˜ linked acid dipeptidase (NAALADase) activity and a pteroyl poly-γ-glutamyl carboxypeptidase (folate hydrolase) activity. On the basis of preliminary data, the ideal TG prodrug should consist of either an aspartate or glutamate containing TG analog coupled via to a peptide containing a series of α- and γ-linked glutamates and ending in an α-linked aspartyl-glutamate "cap". This substrate would be readily cleaved by PSMA but would be stable to hydrolysis by proteases such as gamma-glutamyl hydrolase present in serum and extracellular fluid of some normal tissue types.

15. SUBJECT TERMS
Prodrug, PSMA

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					USAMRMC
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	24	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

.

Table of Contents

.

Introduction:

The majority of our present chemotherapeutic agents only kill cells effectively when they are proliferating; this may explain why these agents have been of such limited success in patients. In contrast to these ineffective agents, we have chemically modified a plant toxin, Thapsigargin (TG), to produce primary amine-containing analogs that are potent, cell proliferation independent, inducers of apoptosis in prostate cancer cells. These TG-analogs, however, are not prostate cancer-specific cytotoxins. Therefore, the hypothesis of this proposal is that a potent TG analog can be converted to inactive prodrugs by coupling to a peptide carrier that is a substrate for Prostate Specific Membrane Antigen (PSMA). In this way the inactive prodrugs can be efficiently converted back to active killing drugs only by the enzymatic activity of PSMA. Since PSMA is expressed in high levels only by prostate cancer cells and not by normal cells, this should allow specific targeting of the TG-analog's killing ability to prostate cancer cells.

Two enzymatic activities for PSMA have been described: an N-acetyl-a linked acid dipeptidase (NAALADase) activity and a pteroyl poly-g-glutamyl carboxypeptidase (folate hydrolase) activity. On the basis of preliminary data, the ideal TG prodrug should consist of either an aspartate or glutamate containing TG analog coupled via to a peptide containing a series of a- and g-linked glutamates and ending in an alpha-linked aspartyl-glutamate "cap". This substrate would be readily cleaved by PSMA but would be stable to hydrolysis by gamma-glutamyl hydrolase (GGH) present in serum and extracellular fluid of some normal tissue types.

Therefore, the specific aims of the study are: (1) to synthesize a series of aspartate or glutamate containing TG analog and characterize their cytotoxicity against human prostate cancer cell lines (2) to synthesize prodrugs by chemically linking the aspartyl and glutamyl containing TG analogs to peptide substrates that require hydrolysis by both the NAALADase activity and folate hydrolase of PSMA. These prodrugs will be assayed for in vitro cytotoxicity against PSMA-producing and non-producing cell lines to determine which of these prodrugs is the best lead compound for future in vivo animal studies; (3) to determine the in vivo efficacy and toxicity of the best (i.e. lead) PSMA prodrug (based upon comparison of the in vitro potency and specificity) against PSMA-producing human prostate cancer xenografts; (4) to develop a molecular model of the PSMA catalytic site and compare binding of peptide substrates tested to date in order to predict optimal PSMA peptide substrate.

.

BODY:

PSMA is a 100 kDa prostate epithelial cell type II transmembrane glycoprotein that was originally isolated from a cDNA library from the androgen responsive LNCaP human prostate cancer cell line. Using a series of monoclonal antibodies, several groups have characterized PSMA expression in various normal and tumor tissues. These studies demonstrated that PSMA is expressed in high levels by both normal and malignant prostate tissue. Low-level expression was only seen in the lumen of the small intestine and the proximal tubules of the kidney. In addition, while normal vascular endothelial cells are PSMA negative, endothelial cells of the tumor vasculature stain positive for PSMA in a large number of tumor types, while the tumor cells themselves do not express PSMA.

In summary, the aforementioned studies highlight the characteristics of PSMA that make it a suitable target for prostate specific therapy. PSMA expression is highly restricted to prostate tissue with strongest expression in both primary and metastatic prostate cancers. The PSMA protein detectable in prostate cancers is an integral membrane protein and therefore has an extracellular domain that is accessible to agents in the extracellular peritumoral fluid making it possible to target this protein with antibodies and prodrugs. A final interesting aspect of PSMA expression is that the PSMA mRNA is upregulated upon androgen withdrawal. In LNCaP cells androgen has been found to downregulate PSMA expression and in patient specimens an increase in immunohistochemically detectable PSMA expression has been observed following androgen ablative therapy. In contrast, PSA expression is downregulated by androgen deprivation. Therefore, PSMA should be readily targetable in the majority of hormone refractory patients because PSMA levels are expected to remain high following androgen ablation.

Two discrete enzymatic functions for PSMA have been described. Initially, Carter et al demonstrated that PSMA possesses the hydrolytic properties of an N-acetylated α-linked acidic dipeptidase (NAALADase). NAALADase is a membrane hydrolase activity that is able to hydrolyze the neuropeptide N-acetyl-*l*-aspartyl-*l*-glutamate (NAAG) to yield the neurotransmitter glutamate and N-acetyl-aspartate. In addition to the NAALADase activity, PSMA also functions as a pteroyl poly-γ-glutamyl carboxypeptidase (folate hydrolase). PSMA exhibits exopeptidase activity and is able to progressively hydrolyze γ-glutamyl linkages of both poly-γ-glutamated folates and methotrexate analogs with varying length glutamate chains. In our proposal, we outline an approach that would take advantage of both the prostate specific expression of the PSMA protein in men and its unique NAALADase and folate hydrolase activities.

A successful PSMA activated prodrug must pass three critical tests. The prodrug must be cleaved by PSMA, it must not be toxic to PSMA-negative cells and its toxicity to PSMA positive cells must be secondary to activation specifically by PSMA. In order to rapidly screen a larger number of prodrugs for PSMA activity and specificity, we previously synthesized a series of analogs based on methotrexate consisting of the pteridine ring-para-aminobenzoic acid (APA) portion of methotrexate coupled to a variety of peptides. This strategy was utilized for several reasons. The coupling of APA to the N-terminal amine of gamma-linked polyglutamates does not inhibit sequential PSMA hydrolysis. The chemistry to produce these analogs has already been described and the large quantities of the inexpensive APA precursor are available. The APA molecule has an absorbance at 340 nm and hydrolysis of prodrugs can be readily followed by HPLC analysis. Finally, APA-Glu (i.e. methotrexate) is cytotoxic at nanomolar concentrations and therefore activation of prodrugs in vitro can be easily assessed using growth inhibition and clonogenic survival assays. This strategy, therefore, was used to identify peptides that were selectively hydrolyzed by PSMA while at the same time remaining stable to hydrolysis in human plasma.

These studies with methotrexate-based peptides have delineated the limited range of substrates that can be hydrolyzed by PSMA. The only alpha-linked methotrexate substrate (i.e. substrate for PSMA's NAALADase activity) that was hydrolyzed by PSMA was APA-Asp-Glu. This substrate was also stable to hydrolysis by gamma-glutamyl hydrolase (GGH) and other

.

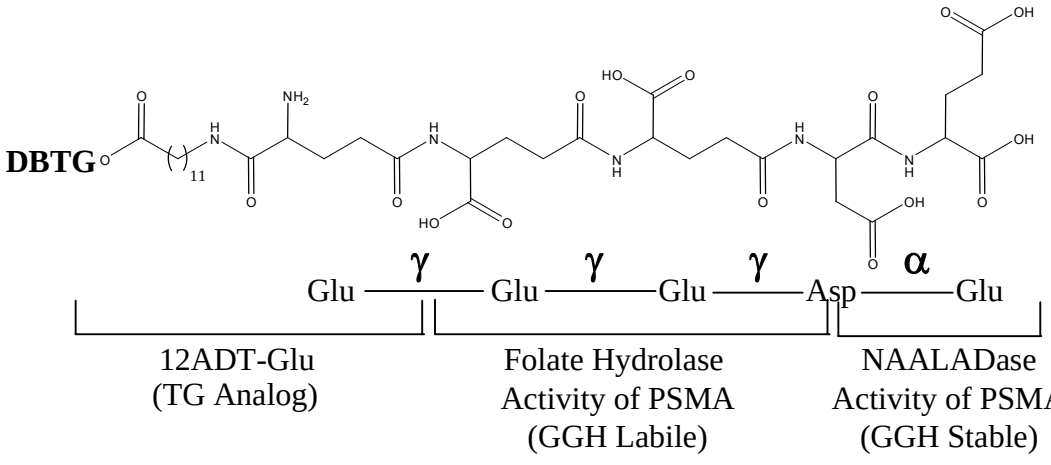
protease activity present in the serum, table 1. These previous studies also demonstrated the enhanced ability of PSMA to hydrolyze a variety of γ-linked substrates. These γ-linked substrates, however, were not specific for PSMA and were readily hydrolyzed in human plasma. On this basis, we reasoned that the ideal substrate would take advantage of the dual ability of PSMA to hydrolyze certain alpha and gamma linkages between aspartyl and glutamyl residues. Thus, the ideal substrate should incorporate the specificity of the α-linkage with the enhanced efficiency of the γ-linkage. The longer length, negatively charged, substrates would serve two additional purposes: first, they help to make the highly lipophilic more toxins like TG analogs more water soluble; second, the highly charged prodrug will be less likely to cross the plasma membrane, further limiting non-specific cytotoxicity, figure 1.

Table 1. Hydrolysis of PSMA-substrates by purified PSMA and stability in plasma

	% PSMA[2] Hydrolysis		18 h Incubation Human Plasma		18 h Incubation Mouse Plasma
Substrate	4 h	24h	% APA-Glu or Asp	% Prodrug Remaining[1]	% APA-Glu or Asp
α-linked					
APA-Asp-Glu	0	62	1	99	ND
APA-Glu-Glu	0	0	ND	ND	ND
γ-linked					
APA-Glu-Glu-Glu-Glu-Asp	18	86	9	20	72
APA-Glu-Glu-Glu-Glu-Gln	48	92	6	26	62
APA-Glu-Glu-Glu-Glu-Glu	50	96	11	11	78

[1]% Prodrug Remaining equals peak area of starting material/total peak area (sum of prodrug and intermediate peaks). [2] % complete hydrolysis to APA-Asp or APA-Glu by purified PSMA. ND= not done

Using this rationale, additional substrates were synthesized in which a PSMA-hydrolyzable a-linked dipeptide "cap" is introduced that is not a substrate for GGH in order to produce more specific PSMA substrates. One of these substrates APA-Glu*Glu*Glu*Asp-Glu was a less efficient PSMA substrate but showed enhanced stability in human serum. A second substrate APA-Glu*Glu*Glu*Asp-Gln was a poor substrate for PSMA, although it had even more enhanced stability in serum. Finally, a substrate containing two α-linkages and two γ-linkages, APA-Asp-Glu*Glu*Asp-Glu was a better PSMA substrate and was completely stable to hydrolysis in human and mouse plasma, table 1. These combination alpha- and gamma-linked PSMA substrates possess the best combination of efficiency and specificity and these substrates will therefore be used to create prodrugs described below.



.

Figure 1. Example of PSMA prodrug containing α-and γ-linked amino acids and TG analog 12ADT-Glu.

Rationale for use of Thapsigargin in the treatment of Prostate Cancer

Thapsigargin (TG) is a sesquiterpene γ-lactone isolated from the root of the umbelliferous plant, *Thapsia garganica*, figure 2. TG has been shown to increase intracellular Ca^{2+} and induce programmed cell death in prostate cancer cell lines as well as a host of other normal and malignant cell types. TG induces programmed cell death of all rapidly proliferating prostate cancer cell lines. Unlike standard antiproliferative agents such as 5-FU or doxorubicin, TG can also induce apoptosis in non-proliferating, G_0 arrested prostate cancer cells. TG represents an excellent choice for treatment of prostate cancer because of its ability to kill these cells in a proliferation-independent manner. This ability to induce apoptosis in a proliferation-independent manner is particularly advantageous for the treatment of prostate cancer since these tumors have been demonstrated to have a remarkably low rate of proliferation (i.e. < 5% cells proliferating/day). Unfortunately, while TG is highly effective in inducing the proliferation independent programmed cell death of androgen independent prostate cancer cells, it is not cell type specific and is sparingly water soluble due to its high lipophilicity. In order to target TG's cytotoxicity specifically to prostate cancer cells systemically, TG has been chemically modified to produce a primary amine containing analog that can be coupled to a water-soluble peptide carrier. This modification involves the introduction of a primary amine containing side chain into the TG molecule that can be coupled via a peptide bond to the carboxyl group of amino acid. Such a TG analog can be coupled to the alpha (Asp or Glu), beta (Asp) or gamma(Glu) carboxyl of Asp or Glu residues to produce prodrugs that can be targeted specifically to metastatic deposits of androgen independent prostate cancer producing enzymatically active PSMA, figure 3.

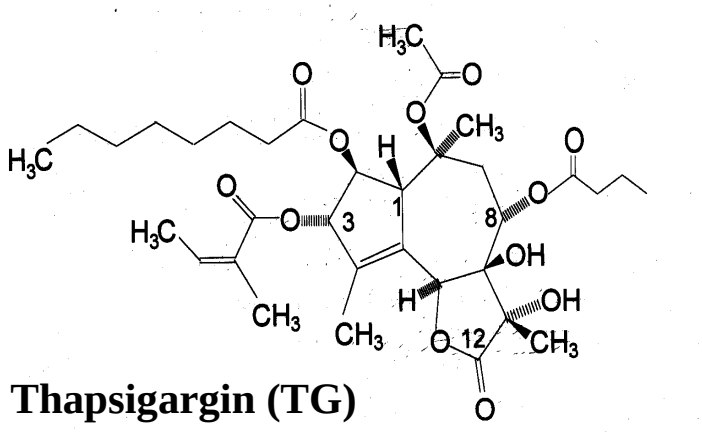


Figure 2. Chemical structure of thapsigargin

Progress over the funding period.

Task 1:To synthesize and characterize the cytotoxicity of a series of Asp- or Glu-containing TG analogs.

(1-12 months).

.

In **Task 1** of the proposal we proposed the synthesis of a series of TG analogs that could be chemically linked to PSMA specific peptides. Because PSMA is an exopeptidase, hydrolytic processing of any prodrug will result in an end product consisting of a cytotoxin coupled to an acidic amino acid, most likely glutamate or aspartate. Therefore, while a TG analog can be potentially targeted using this prodrug approach, preferred analogs would be those that incorporate glutamic or aspartic acid into their structure and still maintain their cytotoxicity. In previous years of funding we accomplished this task by synthesizing the analogs 12ADT-Glu, 12ADT*Glu and 12ADT$Asp and demonstrated that they were potent cytotoxins with IC_{50} values against cancer cell lines of < 100 nM, table 2. These analogs were not selective to prostate cancer cells as they were cytotoxic to both LNCaP human prostate cancer cells and TSU human bladder cancer cells. As part of the evaluation of these analogs we also demonstrated that our lead toxin, 12ADT$Asp was able to inhibit the SERCA pump with a potency ~ equal to thapsigargin, table 3.

On the basis of these studies we have completed Task 1 as outlined in the Statement of Work.

Table 2. IC50 values for inhibition of LNCaP (human prostate) and TSU (human bladder) cancers

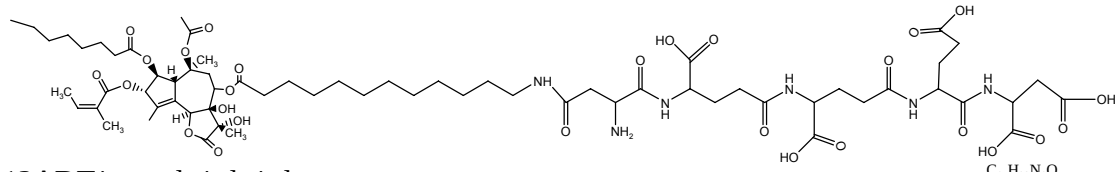
Analog	LNCaP (PSMA +)	TSU (PSMA -)
Thapsigargin	27±6	30±8
12ADT$Asp	40±12	72±26
12ADT-Glu	50±6	60±20
12ADT*Glu	60±19	60±6

α-linkage denoted by hyphen (-) , γ-linkage by star (*) and β by ($)

Task 2: To synthesize prodrugs by chemically linking the TG analogs to peptide substrates that require hydrolysis by both the NAALADase activity and the folate hydrolase activity of PSMA. (6-24 months)

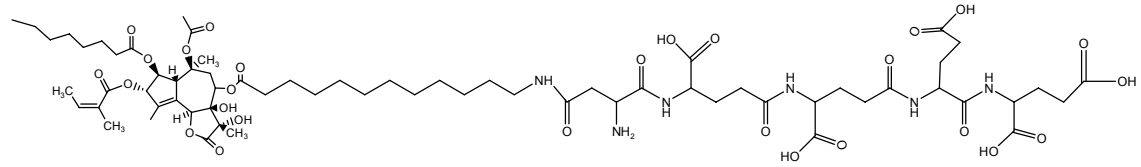
The goal of **Task 2** of the proposal was to synthesize prodrugs by chemically linking the TG analogs to peptide substrates that require hydrolysis by either NAALADase activity alone or combined NAALADase and folate hydrolase activity of PSMA. Over the entire funding periodwe synthesized a series of PSMA prodrugs, and characterized each on the basis of PSMA hydrolysis, plasma stability and selective cytotoxicity to PSMA producing cells. Examples of some of these prodrugs are depicted in figure 3.

.



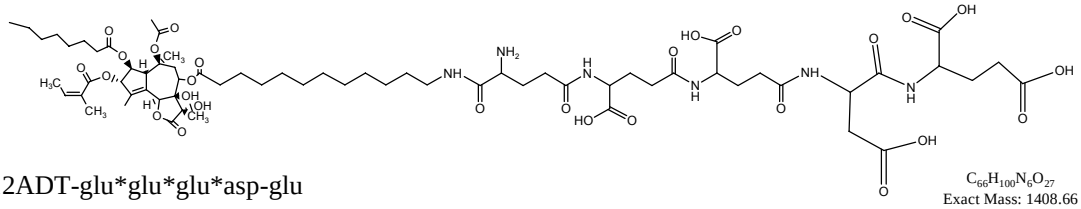
12ADT*asp-glu*glu*glu-asp

$C_{65}H_{98}N_6O_{27}$
Exact Mass: 1394.65
Mol. Wt.: 1395.50
C, 55.94; H, 7.08; N, 6.02; O, 30.96



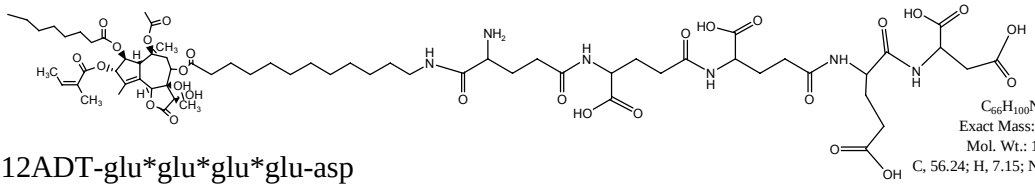
12ADT*asp-glu*glu*glu-glu

$C_{66}H_{100}N_6O_{27}$
Exact Mass: 1408.66
Mol. Wt.: 1409.52
C, 56.24; H, 7.15; N, 5.96; O, 30.65



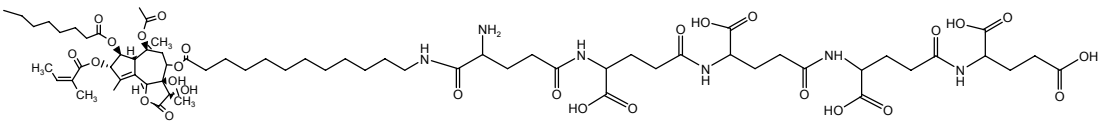
12ADT-glu*glu*glu*asp-glu

$C_{66}H_{100}N_6O_{27}$
Exact Mass: 1408.66
Mol. Wt.: 1409.52
C, 56.24; H, 7.15; N, 5.96; O, 30.65



12ADT-glu*glu*glu*glu-asp

$C_{66}H_{100}N_6O_{27}$
Exact Mass: 1408.66
Mol. Wt.: 1409.52
C, 56.24; H, 7.15; N, 5.96; O, 30.65



12ADT-glu*glu*glu*glu*glu

$C_{67}H_{102}N_6O_{27}$
Exact Mass: 1422.68
Mol. Wt.: 1423.55
C, 56.53; H, 7.22; N, 5.90; O, 30.35

Figure 3. Chemical structures of additional putative PSMA-activated TG prodrugs

.

To assess PSMA hydrolysis, prodrugs were incubated with PSMA and hydrolysis products were determined by HPLC analysis. Peak identities were confirmed by mass spectroscopy. To further assess specificity, all prodrugs were incubated in human plasma for 48 h at which time no significant proteolysis of any of the analogs was observed. From these analyses the prodrug that was most efficiently hydrolyzed by PSMA while maintaining stability in plasma were 12adt*asp-glu and 12adt*asp-glu*glu*glu*glu

In clonal survival assays 12Adt*asp-glu (Amm2) was cytotoxic to PSMA producing C42B cells and this cytotoxicity was completely inhibited by 2-(phosphonomethyl)pentanedioic) acid (2-PMPA), a potent competitive inhibitor of PSMA's enzymatic activity. These results demonstrated that increased toxicity of 12Adt*asp-glu was dependent on presence of enzymatically active PSMA in this cell line. The toxicity of all prodrugs were further studied in non-PSMA producing TSU and compared to the PSMA-producing LNCaP cell line. All of the tested prodrugs that were cleaved by PSMA showed significant reduction in LNCaP cell growth in these assays over the dose range tested. As a control, a prodrug that was not cleaved by PSMA was evaluated. This prodrug was relatively inactive against PSMA-producing LNCaP and equitoxic to LNCaP and TSU cells demonstrating a requirement for PSMA for activation, table 3. All prodrugs were less potent against non-PSMA expressing TSU cells (table 3). These data depict that all prodrugs are relatively "inactivate" in the absence of PSMA. The IC50 values (prodrug concentration required to inhibit colony survival by 50%) were calculated (table 3). IC50 values against TSU were in the micromolar range while values against LNCaP were in the nanomolar range. Overall the prodrugs were ~15-60 fold more active in vitro against PSMA producing cells, table 3.

Table 3. IC_{50} values for selected prodrugs against PSMA-producing LNCaP cells and non-PSMA producing TSU cells in clonal survival assays.

Prodrug/drug	IC_{50} Values (nM)		
	TSU	LNCaP	Fold Diff
12ADT$asp	40 ± 12	72 ± 26	2
12ADT$asp-glu	7629 ± 1008	251 ± 6	30
12ADT$asp-glu*glu*asp-glu	10816 ± 1141	384 ± 36	28
12ADT-glu*glu*glu*glu*glu	8541±857	239 ± 8	35
12ADT-glu*glu*glu*asp-glu	8507 ± 800	344 ± 9	25
12ADT$asp-glu*glu*glu*glu	10964 ± 400	191 ± 29	57
12ADT$asp-glu*glu*glu-asp (Not cleaved by PSMA)	10700 ±400	5351 ± 600	2

.

After having established that all prodrugs show diminished potency against non-PSMA producing cell lines, it was critical to determine whether this decrease was due to the absence of PSMA and poor cell penetration by the peptide-based prodrug. Alternatively, the presence of the peptide carrier is not sufficient to prevent cell penetration and the decreased potency in non-PSMA producing cells, therefore is due to both lack of PSMA hydrolysis of the prodrug and the poor inhibition of the SERCA pump by intracellular prodrug. To address these possibilities, microsomes were isolated by differential centrifugation from rabbit muscle, which are very rich in SERCA. An enzyme-coupled assay utilizing auxiliary and indicator reactions was employed to quantify Ca^{2+}ATPase activity in microsomes. Ca^{2+}ATPase activity in isolated SERCA decreased with increase in inhibitor (drug/prodrug/TG) concentration. The IC50 values expressed in nM/mg SR protein (inhibitor concentration required to decrease CaATPAse activity to 50%) were determined for various prodrug analogues and normalized to TG or its drug analog 12Adt*asp (table 3). The longer prodrugs exemplified by 12Adt-glu*glu*glu* asp- glu (Amm7) and 12Adt*asp-glu*glu* asp- glu (Amm1) had the lowest potency against SERCA. 12Adt*asp-glu (Amm2) showed higher potency than the pentamers but lower potency than the drug 12Adt*asp. The ultimate drug 12Adt*asp was equipotent to the natural SERCA inhibitor TG. Although IC_{50} values increased with chain length in the SERCA assays, the magnitude (~6nM vs. 25nM) is small compared to the difference between IC_{50} values in clonal assays in non-PSMA and PSMA producing lines (nM vs. uM range) (table 2). These results suggest that the fold differences in potency of these prodrugs are due to prodrug processing by PSMA-producing cells. In contrast, the peptide prevents non-specific cell penetration and therefore less cytotoxicity against non-PSMA producing cells.

Table 4. Inhibition of SERCA pump by TG and PSMA-activated TG prodrugs

Drug	IC50 nM/mg SR protein	IC50 Relative to TG	IC50 Relative to 12Adt*Asp
TG	**6.6 +/- 2.3**	**1**	**0.9**
12Adt*Asp	**7.5 +/- 2.2**	**1.2**	**1**
Amm1	**24.3 +/-1.9**	**3.8**	**3.2**
Amm2	**15.3 +/- 1.7**	**2.4**	**2**
Amm7	**26.1 +/- 3.7**	**4.1**	**3.5**

The final objective of Task 2 was to assess stability of these prodrugs in human and mouse plasma. For all of these studies prodrugs were incubated in plasma for 24 hrs and stability assessed by HPLC analysis for release of hydrolysis products. All of the PSMA prodrugs were stable in human and mouse plasma under these conditions (i.e. <1% production of free 12ADT-Asp) (data not shown). On the basis of these studies we have completed Task 1 as outlined in the Statement of Work.

Task 3: To determine the in vivo efficacy of the best (i.e. lead) PSMA prodrug based upon comparison of the potency and specificity data of Task 2. (18-36 months)

The goal of **Task 3** was to determine the in vivo efficacy of the best (i.e. lead) PSMA prodrug based upon comparison of the potency and specificity data of Task 2. On the basis of the data from studies in task 2, the lead PSMA prodrugs was deemed to be 12ADT*Asp-Glu*Glu*Glu*Glu. This prodrug was synthesized in sufficient quantity to perform toxicity, pharmacokinetic and

.

efficacy studies. Toxicity studies revealed that mice could tolerate repeat injections of 1 μmole of the prodrug compounds x 3 and daily injections of 0.3 μmoles for 10 days. The LD50 (i.e. dose that caused death of 50% of animals) for this compound was 3.0 μmoles. On this basis, pharmacokinetic studies were performed with both prodrugs. In these studies animals were given a single intravenous dose of 1.2 μmoles of the lead prodrug. At various time points animals were bled and serum obtained for analysis. In the initial application we described use of 3H labeled analog. However, over the course of the grant period we gained access to an liquid chromatography tandem mass spectrometer (LC-MS-MS) that allowed us to determine tissue levels without the need for radioactive compound. This equipment was available to us on the basis that we pay a portion of the yearly service contract (i.e. $7500/year). Therefore, prodrug levels were determined using LC-MS-MS analysis. From these studies we determined that the half life of 12ADT*Asp-Glu*Glu*Glu*Glu in mouse circulation following a single intravenous dose of 67 mg/kg was 5 hrs, figure 4. While this compound was stable ex vivo in mouse plasma as described in Task 2, it was also stable in mouse circulation in vivo. Overall, less than 1% of the 12ADT*Asp-Glu*Glu*Glu*Glu was converted to the toxic 12ADT*Asp analog, figure 4.

.

In additional studies we used LC-MS to compare levels of prodrug and free 12ADT$Asp in plasma and CWR22RH tumor five days after a single injection of 112 mg/kg prodrug, figure 5. These results demonstrated that, while the prodrug does not accumulate in tumors and is cleared from plasma, the 12ADT-Asp toxin builds up in tumors to high levels and is retained.

Figure 4. Pharmacokinetic analysis of prodrug and 12ADT-Asp levels in mouse circulation following single injection fo 67 mg/kg prodrug . Mice (n=3 per time point) were sacrificed at indicated time post dose and blood levels determined by LC-MS-MS.

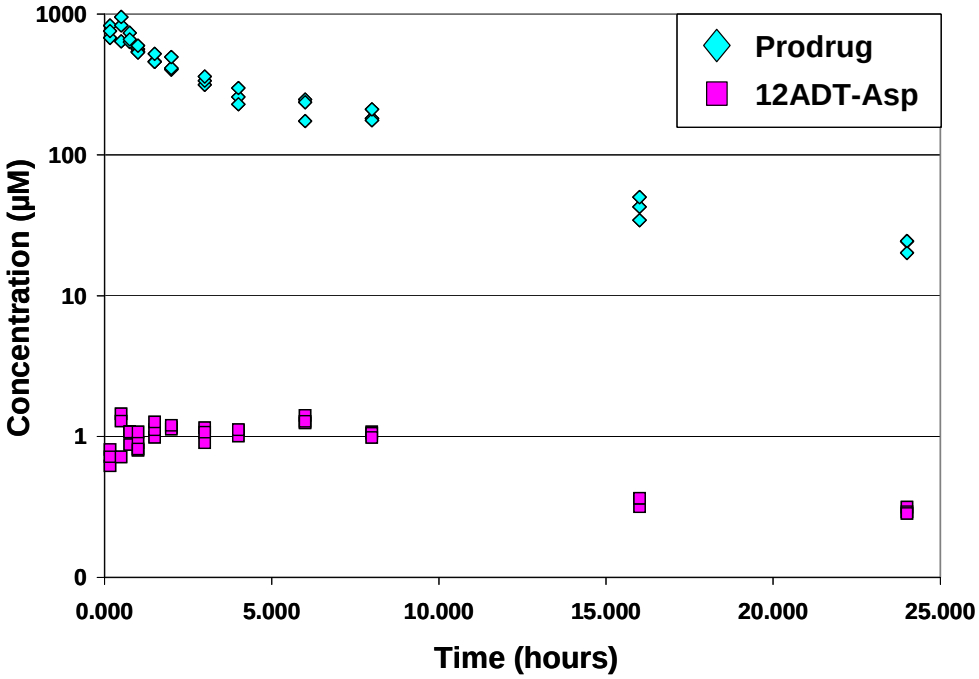
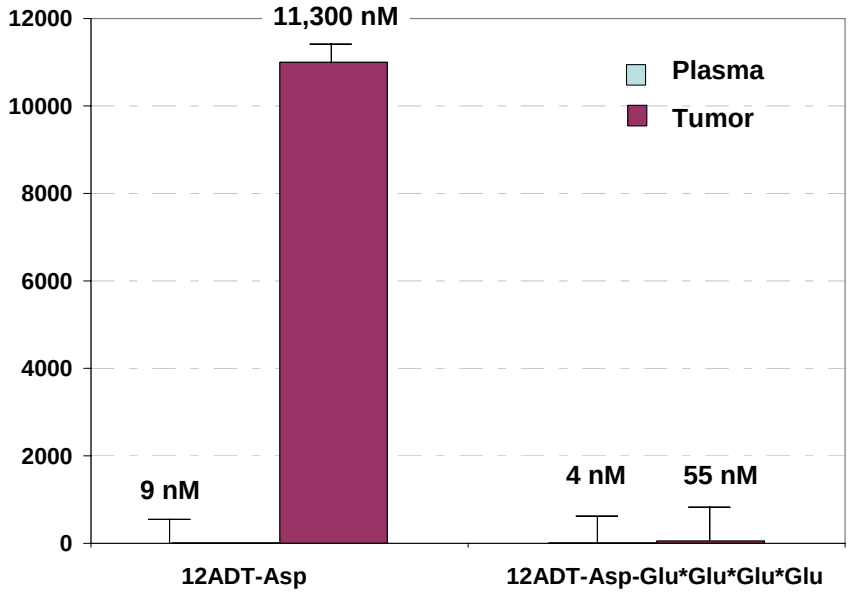


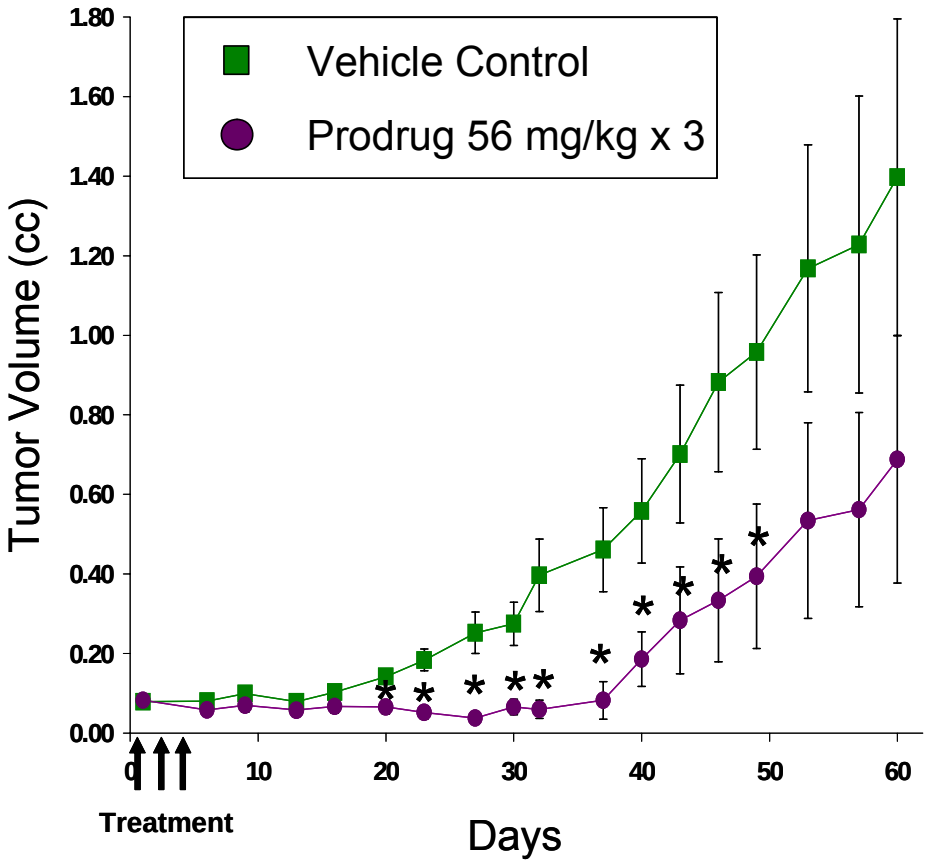
Figure 5. Levels of prodrug and liberated 12ADT-Asp in tumor and plasma 5 days after a single injection of 112 mg/kg PSMA prodrug.



.

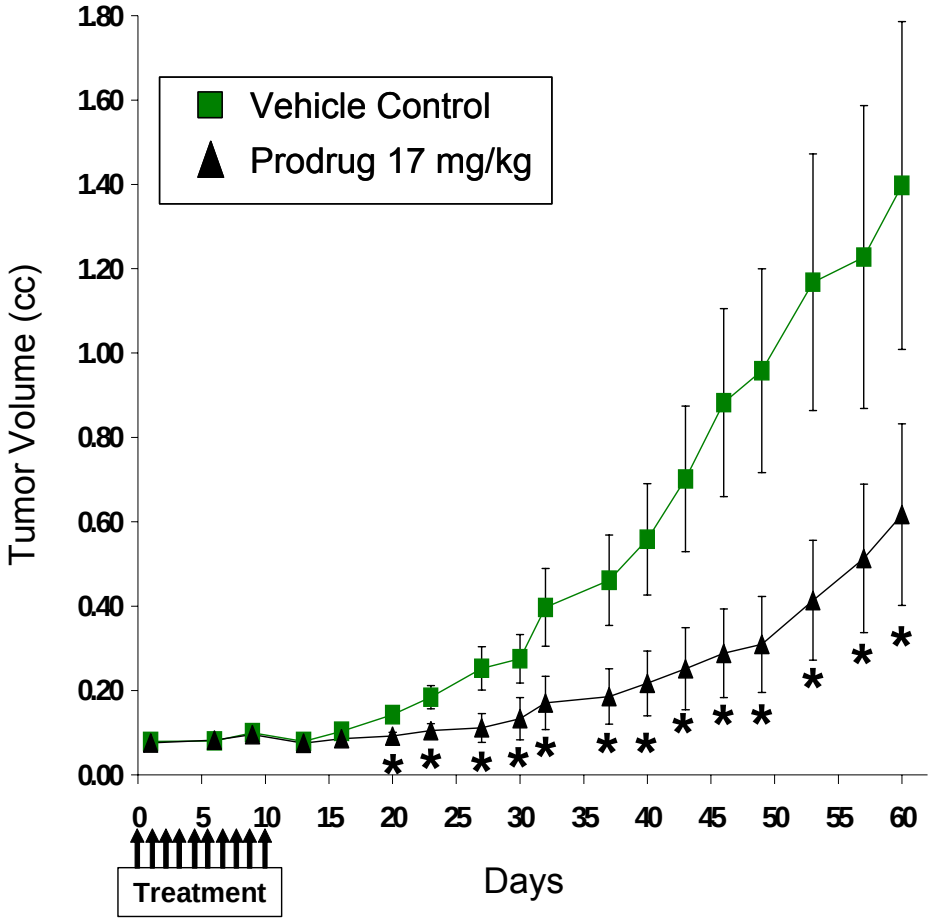
In initial in vivo efficacy studies we evaluated effects of daily intravenous injection of 0.3 μmoles (17 mg/kg)of the prodrug for a total of 7 days on the growth of PSMA-positive LNCaP prostate cancer xenografts. In this initial study, the 12ADT*Asp-Glu*Glu*Glu*Glu was able to inhibit growth by >75% (data not shown). Due to limited supply of each prodrug this study was stopped after one cycle of drug. Subsequently, we synthesized an additional 1 gram of each prodrug to allow for longer dosing intervals. In a second in vivo study we used a subclone of the CWR22 human prostate cancer xenograft line. CWR22 is an androgen dependent model that must be serially passaged as a xenograft in nude mice. We have been able to generate an androgen independent clone of this line which grows well in castrated mice. We demonstrated that this CWR22H clone makes much higher levels of PSMA then LNCaP in vivo. Therefore, this clone was inoculated into nude mice and after two weeks, when tumors were just palpable, treatment with two dose levels of prodrugs was initiated. Choice of dose levels was based on initial toxicity studies. Animals received either 10 daily injections of 0.3 μmoles (i.e. 17 mg/kg) of the prodrug or 3 days of 1.0 μmoles (56 mg/kg). All animals received the same total dose of prodrug over the course of the experiment. Animals received a single cycle . Results are summarized in figure 6-8. The12ADT*Asp-Glu*Glu*Glu*Glu produced tumor regressions and sustained inhibition of growth at both dose levels. No toxic deaths were observed in any of the treatment groups. Animals treated with 56 mg/kg x 3 doses had transient weight loss but returned to baseline weight by end of the observation period, figure 9. Animals treated with 17 mg/kg x 10 doses had no significant weight loss compared to control, figure 9.

Figure 6. Antitumor efficacy of Prodrug (56 mg/kg x 3 doses indicated by arrows) against CWR22R-H xenografts (* indicates $p<0.05$)



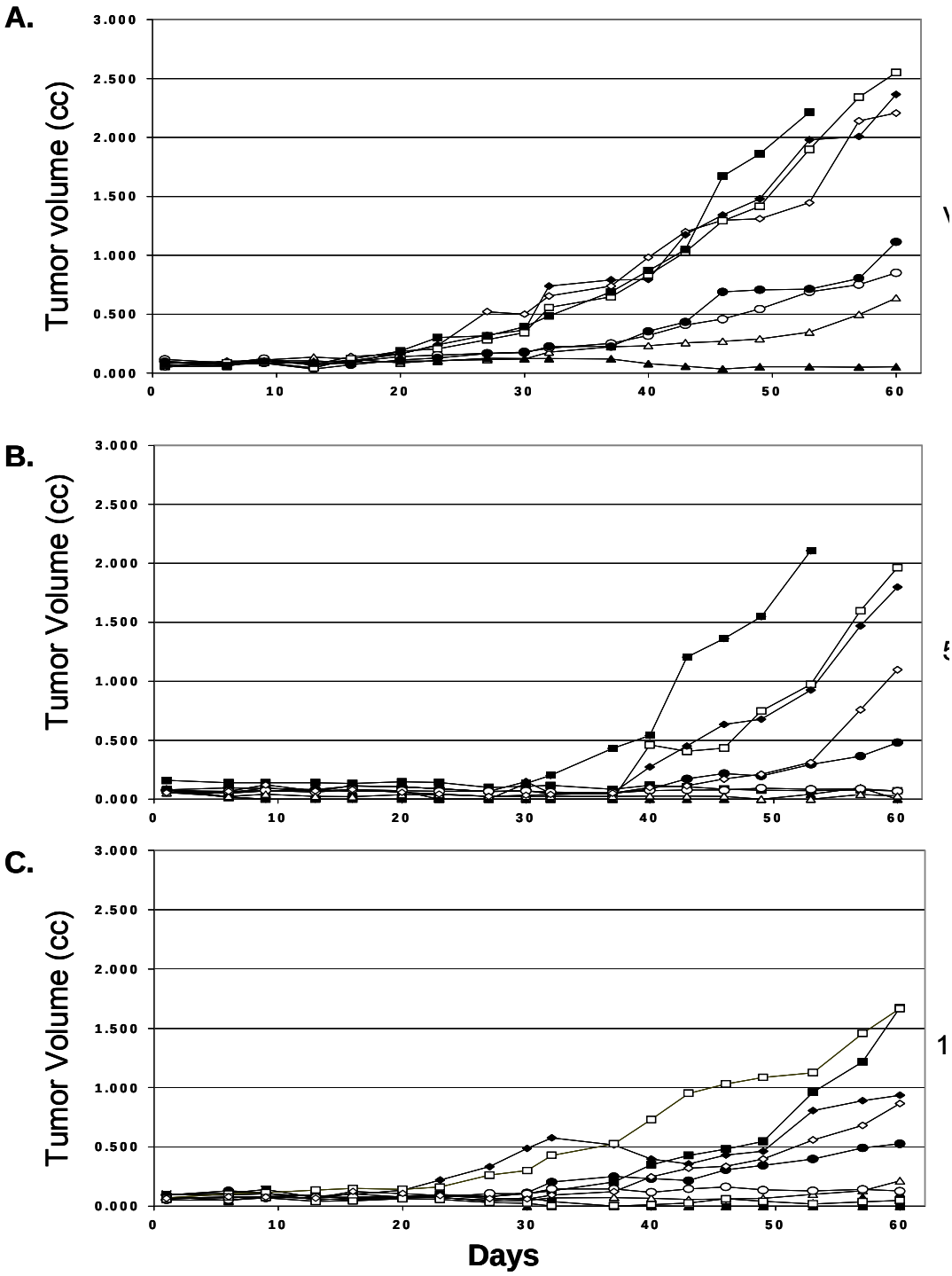
.

Figure 7. Antitumor efficacy of Prodrug (17 mg/kg x 10 doses indicated by arrows) against CWR22R-H xenografts (* indicates $p<0.05$)



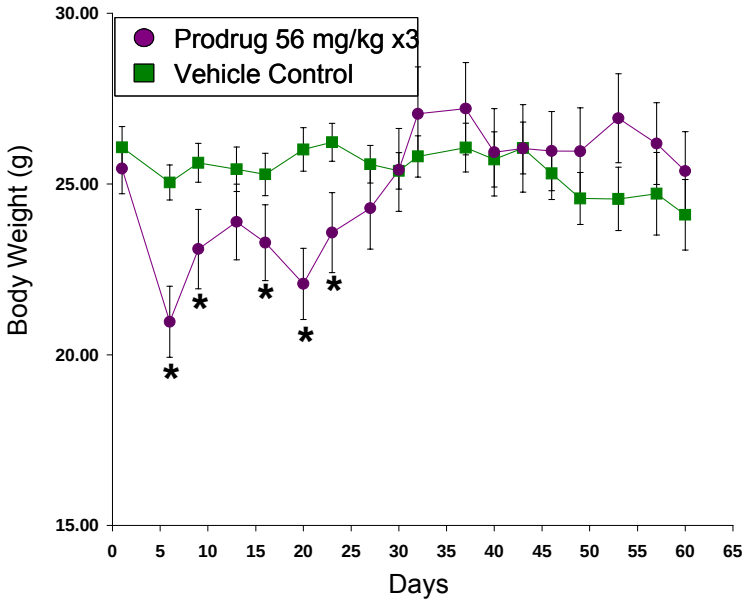
.

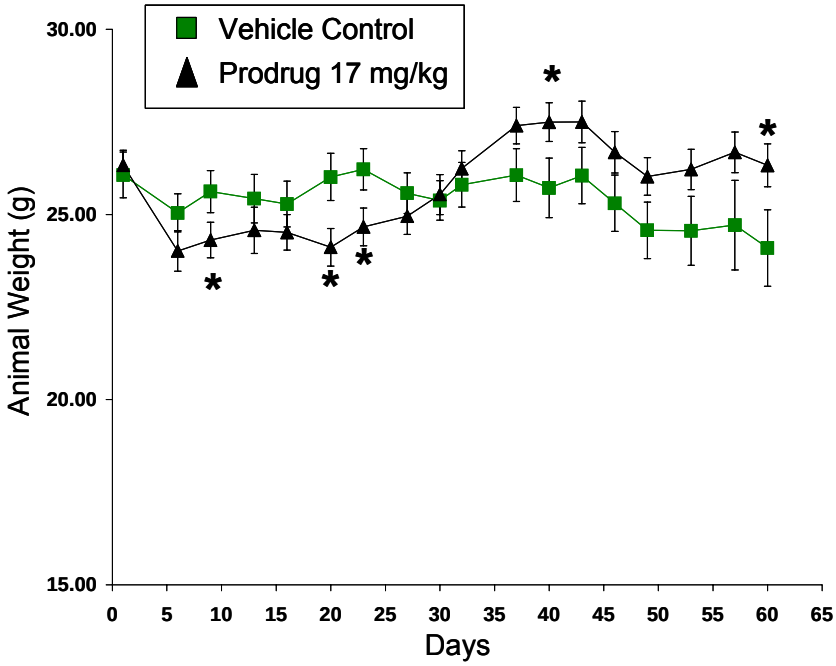
Figure 8. Growth of individual tumors. (A) Vehicle control treated; (B) Prodrug 56 mg/kg x 3 days (C) Prodrug 17 mg/kg x 10 days



.

Figure 9. A) Animal weights following treatment with 3 days of 56 mg/kg Prodrug or 10 days of vehicle control. (* indicates p<0.05) B) Animal weights following treatment with 10 days of 17 mg/kg Prodrug or 10 days of vehicle control. (* indicates p<0.05)





These collective results demonstrate that the PSMA-activated prodrug can be given safely to animals at doses that produce inhibition of tumor growth. On the basis of these studies we have completed Task 1 as outlined in the Statement of Work.

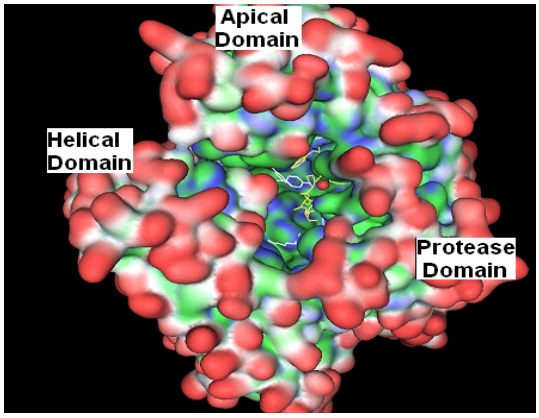
.

Task 4: To develop a molecular model of PSMA Catalytic Site to analyze binding of putative PSMA substrates. (6-24 months).

The goal of this task was to develop a model of the PSMA binding site to define additional prodrug candidates. This task was not completed as Dr. Randy Wadkins, who initially generated the homology model for PSMA described in the grant submission, left Johns Hopkins due to lack of funding. No money was budgeted in this grant for this aim for equipment or supplies. In addition, no money was budgeted for Dr. Wadkins salary.
Subsequent to Dr. Wadkins leaving, our group recruited Pratap Singh, a graduate student in the Chemical and Biomolecular Engineering Department at Johns Hopkins. Dr. Singh had prior expertise in computer modeling. In 2005 (i.e. after grant funding period for DAMD 17-02-1-0076), Dr. Singh and our group published studies analyzing binding of thapsigargin analogs to a model of the SERCA pump that was developed by Dr. Singh based on publication of the SERCA crystal structure bound to thapsigargin. (Singh P, Mhaka AM, Christensen SB, Gray JJ, Denmeade SR, Isaacs JT Applying linear interaction energy method for rational design of noncompetitive allosteric inhibitors of the sarco- and endoplasmic reticulum calcium-ATPase. J Med Chem. 2005;48:3005-14).

Subsequently in 2005, the crystal structure of PSMA was also published. Dr. Singh has used this crystal structure to generate a model of the binding site for PSMA, figure 10. We have used this model to evaluate PSMA inhibitory peptides and can also use this model to identify PSMA substrates.

Figure 10. Space filling model of PSMA catalytic site with PSMA binding peptide within cavity.



.

KEY RESEARCH ACCOMPLISHMENTS:

1. Synthesized a series of PSMA-activated thapsigargin peptide prodrugs.

2. Demonstrated that several of the TG prodrugs are efficiently hydrolyzed by PSMA.

3. Demonstrated that these PSMA-activated TG prodrugs are stable to hydrolysis in human and mouse plasma

4. Determined cytotoxicity of these prodrugs against PSMA-producing and PSMA non-producing human cancer cell lines and found ~15-57-fold difference in cytotoxicity in cells that produced PSMA vs. cells that did not.

5. Synthesized sufficient quantities of lead PSMA-activated prodrugs to initiate in vivo toxicology and antitumor efficacy studies.

6. Performed pharmacokinetic studies of lead prodrug 12ADT*Asp-Glu*Glu*Glu*Glu and determined half-life and stability in whole animal

7. Demonstrated significant antitumor efficacy of 12ADT*Asp-Glu*Glu*Glu*Glu given daily x 3 and daily x 10.

.

REPORTABLE OUTCOMES:

Presentations:

"Enzyme activation of Thapsigargin Prodrugs by Prostate-Specific Membrane Antigen as Targeted Therapy for Prostate Cancer". Poster presentation at Fellow's Research Day, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, MD, 2002.
Graduate student Annastasiah Mhaka winner of prize for "Best Translational Research Presentation".

"Enzyme activation of Prodrugs by Prostate-Specific Membrane Antigen". Poster presentation at EORTC/American Association of Cancer Research (AACR) meeting, Frankfurt, Germany, 2003

"Targeted Activation of Cytotoxic Prodrugs/Protoxins by Prostate-Specific Proteases" The Brady Urologic Institute Thursday Evening Seminar Series, Johns Hopkins University, Baltimore, MD, 2003.

"Enzyme activation of Prodrugs by Prostate-Specific Membrane Antigen". Poster presentation at American Association of Cancer Research (AACR) meeting, Orlando FL, 2004.

Manuscripts and Abstracts:

1. **Mhaka, A., Denmeade, S. R.,** Yao, W., Isaacs, J. T., Khan S. R. A 5-Fluorodeoxyuridine Prodrug as Targeted Therapy for Prostate Cancer. Bioorg. Medicinal Chemistry 12:2459-2461, 2002.

2. **Mhaka, A.,** Janssen, S., Rosen, M., **Denmeade, S.R**. Enzyme activation of Prodrugs by Prostate-Specific Membrane Antigen. European J Cancer 38, Suppl 7:S36, 2002.

3. **Denmeade, S.R.,** Sokoll, L.J., Dalrymple, S., Rosen, D.M., Gady, A.M., Bruzek, D., Ricklis, R.M., Isaacs, J.T. Dissociation Between Androgen Responsiveness for Malignant Growth vs. Expression of Prostate Specific Differentiation Markers PSA, hK2 and PSMA in Human Prostate Cancer Models. Prostate, 54:249-257, 2003.

4. **Denmeade, S.R.,** Jakobsen, C., Janssen, S., Khan, S.R., Lilja, H., Christensen, S.B. and Isaacs, J.T. Prostate-Specific Antigen (PSA) Activated Thapsigargin Prodrug as Targeted Therapy for Prostate Cancer, J Natl Cancer Inst. 95:990-1000, 2003.

5. **Mhaka A**, Gady AM, Lo K-M, Gillies S, **Denmeade SR**. Use of Methotrexate-Based Peptide Substrates to Characterize the Substrate Specificity of Prostate-Specific Membrane Antigen (PSMA). Cancer Biol Ther. 3:551-8, 2004.

6. Jakobsen CM, **Mhaka A**, Isaacs JT, Christensen SB, Olsen CE, **Denmeade SR**. Thapsigargin Analogs Applicable for Prodrugs Activated by Prostate-Specific Membrane Antigen. Bioor Med Chem, In Press, 2005.

7. Singh P, **Mhaka AM**, Christensen SB, Gray JJ, **Denmeade SR**, Isaacs JT. Applying Linear Interaction Energy Method for rational design of non-competitive allosteric inhibitors of SERCA ATPase. J Med Chem. 2005;48:3005-14.

.

Employment Promotions:

Dr. Denmeade, the PI, was promoted to Associate Professor in June 2004 in part due to work performed during the funding period.

Annastasiah Mhaka completed graduate training and gained employment as a consultant with Acidophil, a biotechnology company focused on development of targeted therapies for prostate cancer.

Trainees Mentored:

Annastasiah Mhaka, a graduate student in the Cell and Molecular Medicine Program at Johns Hopkins received her graduate degree based on studies performed in support of this proposal. Her thesis was entitled "Prostate specific membrane antigen (PSMA): a novel target for prostate cancer prodrug therapy. A manuscript detailing these studies is currently in preparation.

Patent application:

"Prodrug activation by prostate-specific membrane antigen", Inventors S. Denmeade and J. Isaacs (PI) submitted May 2000.

Licenses:

The PSMA-activated prodrug technology developed through this funding has been licensed to GenSpera, Inc. a start-up biotechnology company are undergoing preclinical/clinical development as therapy for advanced prostate cancer.

Employment:

Salary support for Graduate Student Annastasiah Mhaka to carry out experiments outlined in this proposal.

.

CONCLUSIONS:

Over the funding period we have completed tasks 1, 2 and 3 as outlined in our original proposal. We have synthesized and characterized novel cytotoxic analogs of the natural product thapsigargin. These analogs are not prostate cancer specific cytotoxins and, therefore, we devised a method to target these potent toxins to prostate cancer by creating inactive prodrugs that can only be activated by the carboxypeptidase activity of PSMA. We identified a series of PSMA selective peptide substrates and coupled the analogs to them to generate prodrugs. These PSMA-activated prodrugs were characterized on basis of PSMA hydrolysis and stability in human and mouse plasma. Prodrugs that were cleaved by PSMA were selectively toxic (i.e. 15-57 fold) to PSMA-producing prostate cancer cells in vitro. We selected two lead drugs for further in vivo testing and performed toxicity, pharmacokinetics and efficacy studies. We identified one prodrug 12ADT*Asp-Glu*Glu*Glu*Glu that was able to completely inhibit growth of PSMA-producing prostate cancer xenografts with minimal toxicity to the host animal. In ongoing experiments, ~80% of treated animals do not have evidence of tumor after ~ 45 days of observation.

On the basis of these results, the PSMA prodrugs have been licensed to a biotechnology company and are entering clinical development (i.e. GMP production, animal toxicity studies, etc) required prior to IND filing and human trials.

.

REFERENCES

1. Carter RF, Feldman AR, Coyle JT. Prostate-specific membrane antigen is a hydrolase with substrate and pharmacological characteristics of a neuropeptidase. Proc. Natl. Acad. Sci., USA 93:749-753, 1996.
2. Chang SS, Reuter VE, Heston WDW, Bander NH, Grauer LS, Gaudin PB. Five different anti-prostate-specific membrane antigen (PSMA) antibodies confirm PSMA expression in tumor-associated neovasculature. Cancer Res 59:3192-3198, 1999.
3. Christensen, S.B., Andersen, A., Kromann, H., Treiman, M., Tombal, B., Denmeade, S.R, and Isaacs, J.T. Thapsigargin Analogues for Targeting Programmed Death of Androgen-Independent Prostatic Cancer Cells. Bioorg. Medicinal Chemistry, 7:1273-80, 1999.
4. Halsted CH, Ling E-H, Luthi-Carter R, Villanueva JA, Gardner JM, Coyle JT. Folylpoly-γ-glutamate carboxypeptidase from pig jejunum. J Biol Chem 273:20417-20424.
5. Horoszewicz JS, Kawinski E, Murphy JP. Monoclonal antibodies to a new antigenic marker in epithelial cells and serum of prostatic cancer patients. Anticancer Res 7:927-935, 1987.
6. Jackisch, C., Hahm, H., Tombal, B., McCloskey, D., Butash K., Davidson, N., and Denmeade, S.R. Delayed Micromolar Elevation in Intracellular Calcium Precedes Induction of Apoptosis in Thapsigargin-Treated Breast Cancer Cells. In press, July, 2000.
7. Liu H, Moy P, Kim S, Xia Y, Rajasekaran A, Navarro V, Knudsen B, Bander NH. Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium. Cancer Res 57:3629-3634, 1997.
8. Lo K-M, Sudo Y, Chen J, Yue L, Yan L, Kong S-M, Chen L, An Q, Gillies SD. High level expression and characterization of Fc-X fusion proteins in mammalian cells. Protein Engineering 11:495-500, 1998.
9. Luthi-Carter R, Barczak AK, Speno H, Coyle JT. Hydrolysis of the neuropeptide N-acetylaspartylglutamate (NAAG) by cloned human glutamate carboxypeptidaseII. Brain Res 795:341-348, 1998.
10. Luthi-Carter R, Berger, UV, Barczak AK, Enna M, Coyle JT. Isolation and expression of a rat brain cDNA encoding glutamate carboxypeptidase II. Proc Natl Acad Sci USA 95:3215-3220, 1998.
11. Pinto JT, Suffoleto BP, Berzin TM, Qiao CH, Lin S, Tong W, May F, Mukherjee B, Heston WDW. Prostate-specific membrane antigen: A novel folate hydrolase in human prostatic carcinoma cells. Clin Cancer Res 2:1445-1451, 1996.
12. Rhee MS,Lindau-Shepard KJ, Chave J, Galivan JH, Ryan TJ. Characterization of human cellular γ-glutamyl hydrolase. Molec Pharmacol 53:1040-1046, 1998.
13. Silver DA, Pellicer I, Fair WR, Heston WDW, Cordon-Cardo C. Prostate-specific membrane antigen expression in normal and malignant tissues. Clin Cancer Res 3:81-85, 1997.
14. Tiffany CW, Lapidus RG, Merion A, Calvin DC, Slusher BS. Characterization of the enzymatic activity of PSM: Comparison with brain NAALADase. Prostate, 1999.
15. Tombal, B., Denmeade, S.R., and Isaacs, J.T. Assessment and Validation of a Microinjection Method for Kinetic Analysis of $[Ca^{2+}]_i$ in Individual Cells Undergoing Apoptosis. Cell Calcium, 1:19-28, 1999.
16. Wright GL Jr, Grob BM, Haley C, Grossman K, Newhall K, Petrylak D, Troyer J, Konchuba A, Schellhammer PF, Moriarity R. Upregulation of prostate-specific membrane antigen after androgen-deprivation therapy. Urology 48:326-334, 1996.
17. Jakobsen, C.M., Denmeade, S.R., Isaacs, J.T., Gady, A.M., Olsen, C.E., Christensen, S.B. Design, Synthesis and Pharmacological Evaluation of Thapsigargin Analogues for Targeting Apoptosis to Prostatic Cancer Cells. J. Med. Chem., 44: 4696-4703, 2001.
18. Tombal, B., Denmeade, S.R., Gillis, J-M, Isaacs, J.T. A supramicromolar elevation of intracellular free calcium ([Ca2+]i) is consistently required to induce the execution phase of apoptosis. Cell Death Differentiation 9:561-573, 2002.27.

.

19. Mhaka, A., Janssen, S., Rosen, M., Denmeade, S.R. Enzyme activation of Prodrugs by Prostate-Specific Membrane Antigen. European J Cancer 38, Suppl 7:S36, 2002.
20. Denmeade, S.R., Sokoll, L.J., Dalrymple, S., Rosen, D.M., Gady, A.M., Bruzek, D., Ricklis, R.M., Isaacs, J.T. Dissociation Between Androgen Responsiveness for Malignant Growth vs. Expression of Prostate Specific Differentiation Markers PSA, hK2 and PSMA in Human Prostate Cancer Models. Prostate, 54:249-257, 2003.
21. Denmeade, S.R., Jakobsen, C., Janssen, S., Khan, S.R., Lilja, H., Christensen, S.B. and Isaacs, J.T. Prostate-Specific Antigen (PSA) Activated Thapsigargin Prodrug as Targeted Therapy for Prostate Cancer, J Natl Cancer Inst. 95:990-1000, 2003.
22. Mhaka A, Gady AM, Lo K-M, Gillies S, Denmeade SR. Use of Methotrexate-Based Peptide Substrates to Characterize the Substrate Specificity of Prostate-Specific Membrane Antigen (PSMA). Cancer Biol Ther. 2004;3:551-8.
23. Jakobsen CM, **Mhaka A**, Isaacs JT, Christensen SB, Olsen CE, **Denmeade SR**. Thapsigargin Analogs Applicable for Prodrugs Activated by Prostate-Specific Membrane Antigen. Bioor Med Chem, In Press, 2005.
24. Singh P, **Mhaka AM**, Christensen SB, Gray JJ, **Denmeade SR**, Isaacs JT. Applying Linear Interaction Energy Method for rational design of non-competitive allosteric inhibitors of SERCA ATPase. J Med Chem, In press, 2005.